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Evaluation of the binding of perfluorinated compound to pepsin: Spectroscopic analysis and molecular docking

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HIGHLIGHTS

- The interaction mechanism between pepsin and perfluorinated chemicals (PFOA, PFNA) was investigated *in vitro*.
- The binding affinity of PFOA/PFNA with pepsin was explored detailed.
- The conformation of pepsin has been changed after PFOA/PFNA binding.

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ABSTRACT

In this paper, we investigated the binding mode of perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA) to pepsin using spectroscopies and molecular docking methods. Fluorescence quenching study indicated that their different ability to bind with pepsin. Meanwhile, time-resolved fluorescence measurements established that PFOA and PFNA quenched the fluorescence intensity of pepsin through the mechanism of static quenching. The thermodynamic parameters showed that hydrophobic forces were the main interactions. Furthermore, UV-vis, FTIR, three-dimensional fluorescence and molecular docking result indicated that PFCs impact the conformation of pepsin and PFOA was more toxic than PFNA. The conformational transformation of PFOA/PFNA-pepsin was confirmed through the quantitative analysis of the CD spectra. The present studies offer the theory evidence to analyze environmental safety and biosecurity of PFCs on proteases.

1. Introduction

Perfluorinated chemicals (PFCs) have faced increasing scrutiny as an environmental pollutant due to their inertness to environment and biological degradation (Giesy and Kannan, 2001). PFCs occur in numerous consumer products such as fast food packaging, paper plates, waxes, polishes, paints, and cleaning products, as well as in some cosmetics, pharmaceuticals, and were identified as global, persistent and bioaccumulative, potentially harmful pollutants. Food and humans are exposed to PFCs from the use of various PFCs-containing products and the intake of contaminated food and environmental media. Numerous studies reported that PFCs have been found to exhibit acute and subchronic toxicity with organisms, such as hepatotoxicity, immunotoxicity and developmental toxicities. The United States Environmental Protection Agency (US EPA) is currently in the process of beginning extra regulatory scrutiny toward to PFCs (Steenland et al., 2009; EPA, 2005).

Perfluorooctanoic acid (PFOA) and Perfluorononanoic acid (PFNA), members of the PFCs family, have been found in the environment and

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consumer products. Food is presently suggested to being an important exposure source for PFCs in humans (Fromme et al., 2009). PFCs are easier to make their way into the body through consumption of food contaminated with PFCs. Currently, it has been reported that the concentration of PFNA in human serum have risen, ranging from 2.15×10^{-4} to 2.47×10^{-2} μM (Calafat et al., 2007a, 2007b). The highest levels being observed in blood samples collected in USA is 17–5100 ng mL^{-1} for PFOA (Gimenez-Bastida et al., 2015). Multiple toxicity studies have found that exposure to high PFOA/PFNA concentrations results in severe effects in the liver, the kidneys, and bile secretion (Kennedy et al., 2004). Furthermore, high levels of PFCs have also been associated with a higher risk of breast cancer (Bonefeld-Jorgensen et al., 2011, 2014). Previous studies focused on the interactions of PFCs with serum albumin at the molecular level. For example, Chen et al. have studied the interaction of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) with bovine serum albumin (Chen et al., 2015). They have concluded that, the binding of PFOS to bovine serum albumin is much stronger than that of PFOA. Zhang's team has studied the interaction of the PFOS to serum albumin and DNA. They found that the non-covalent interactions of PFOS with serum albumin and DNA altered their secondary conformations (Zhang et al., 2009). However, accompanying by fast food packaging, cosmetics, pharmaceuticals, PFCs firstly enter the digestive system and directly bind with some digestion enzymes before they get into the blood plasma and interact with serum albumin. With this as background, a thorough investigation into their interactions with some digestion enzymes is needed.

As a member of digestion enzymes, pepsin is responsible for the most of digestive activities in the stomach (Cole et al., 2000). Pepsin is a small (35 kDa) globular protein with 326 amino acid residues, each of which has two structurally homologous domains (N-terminal domain: residues 1–172 and C-terminal domain: residues 173–326) (Ying et al., 2015). The two domains almost entirely formed by β-sheets. Pepsin belongs to a multi-tryptophan protein that includes five Trp residues (Trp39, Trp141, Trp181, Trp190, and Trp300). Intrinsic fluorescence of pepsin is caused by these Trp residues, and they have been widely applied to the study of conformational change about the pepsin-ligand interaction. Pepsin also contains sixteen tyrosine residues, two of residues—Asp32 and Asp215—are well active in pepsin (Shintani et al., 1997). It is very probable for PFOA/PFNA to interact with pepsin and affect pepsin's activity or even change its physiological function when PFOA/PFNA enters the human digestive system.

Due to the importance of pepsin in the digestive system of human, the binding properties of PFOA/PFNA with pepsin were evaluated by spectroscopic measurements as well as molecular docking. The primary goal of this study is to investigate the effect of PFOA/PFNA on the structural of pepsin and their relationships to human health by various spectroscopic techniques and molecular modeling method. This study helps us to understand the biological toxicity of PFCs on pepsin at the functional macromolecular level, and thus increase the scope for environmental safety of PFOA and PFNA.

2. Materials and methods

2.1. Materials

The pepsin was purchased from Sigma-Aldrich (St. Louis, MO) and was used without further purification. Pepsin solution (3.0×10^{-6} M) was prepared in pH 2.2 citric acid buffer solution (0.025 M, 0.1 M NaCl), which is the most common pH for pepsin digests. PFOA and PFNA were purchased from J&K Scientific Ltd. (Beijing, China). Stock solutions of PFOA and PFNA were prepared to form a 1.0×10^{-3} M solution. The chemical reagents such as citric acid, Na_2HPO_4, NaCl, etc. used in this study were analytical grade.

2.2. Measurements of spectrum

Fluorescence analysis was performed on FP-6500 spectrofluorometer (JASCO, Japan) with temperature maintained by circulating bath. The excitation wavelengths for pepsin were 280 nm. Each spectrum was the average of three scans. The fluorescence intensity will be reduced if inner filter effect is exist. To accurately correct for any inner filter effects in fluorescence spectrum, the following formula was used (Neamtu et al., 2013):

$$F_{cor} = F_{obs} 10^{\frac{A_{ex}+A_{em}}{2}} \quad (1)$$

where F_{cor} is the fluorescence intensity corrected, F_{obs} is the fluorescence intensity calculated in experiment; A_{ex} and A_{em} are the absorption of the system at excitation and emission wavelength, respectively. The intensity of fluorescence used in this paper is the corrected fluorescence intensity.

Three-dimensional fluorescence spectra were performed under the following conditions: the emission wavelength was recorded at 220–600 nm, the excitation wavelength ranged from 220 to 440 nm with an increment of 5 nm and the number of scanning curves was 44.

Fluorescence decay times were recorded on a time-correlated single photon counting FLS920 system from Edinburgh Instruments, with excitation at 280 nm. The data were fitted with a multiexponential decay and χ^2 was less than 1.10.

The UV–Vis absorption measurements were done with Shimadzu double beam spectrophotometer (Model UV 1700) using a cuvette of 1 cm path length. The UV–Vis absorption spectra of pepsin in the absence and presence of PFOA or PFNA were measured at a range of 250–350 nm in the citric acid buffer solution (pH 2.2).

IR spectra were obtained with Tensor 27 FT-IR spectrometer (Bruker, German) equipped with a Germanium attenuated total reflection (ATR) accessory. All spectra were taken via the ATR method with a resolution of 4 cm^{-1} using 64 scans. To obtain the difference spectrum of pepsin, spectra of buffer were collected under the same conditions, and then the absorbance of the buffer was subtracted from the spectra of the sample. The subtraction process follows the principles below: no characteristic peak between 2200 and 1800 cm^{-1} appear, and the curve is flatness (Yue et al., 2014).

CD measurements were carried out with an Applied Photophysics Chirascan spectropolarimeter (Leatherhead, UK). The CD spectra were measured in 190–250 nm using a 0.1 cm quartz cuvette. Each data point is an average of three separate measurements. The secondary structure contents in pepsin were calculated using the CDNN Program (Applied Photophysics Chirascan spectropolarimeter).

Molecular docking studies were performed using AutoDock 4.2 and AutoDock Tools (ADT). The 3D structure of pepsin was obtained from the Protein Data Bank (PDB ID: 5PEP). The Lamarckian genetic algorithm was applied to calculate the possible conformation of the ligand molecule and macromolecule. The docking grid box for PFOA/PFNA was generated with dimensions ($60 \times 60 \times 60$) using Autogrid with a grid spacing of 0.4 Å. Other parameters are set to the default value. Finally, the docking results were illustrated by Discovery Studio Visualizer 4.5 (Accelrys, free version).

3. Result and discussion

3.1. Analysis of fluorescence quenching of pepsin by PFOA/PFNA

The fluorescence emission spectral study is known as a useful method for measuring the mechanism of binding interactions of

protein with small molecules (Lv et al., 2013). The intrinsic fluorescence of pepsin, which comes from 5 tryptophan (Trp) and 16 tyrosine (Tyr) residues, is often used as an endogenous fluorescent probe to analyze the secondary structure of pepsin (Wang and Zhang, 2013). To envision the pepsin-PFCs interactions, we measured fluorescence spectra of pepsin at its native state pH = 2.2. Fig. 1 showed the fluorescence spectra of pepsin alone as well as in the presence of PFOA/PFNA in pH 2.2 buffer. Upon addition of PFOA/PFNA into the solution of pepsin, the fluorescence intensity of pepsin at 344 nm greatly decreased. The change in the intrinsic fluorescence intensity of pepsin was related to the protein conformational transitions when PFOA/PFNA was bound to pepsin. Of the two PFCs, PFNA induced the smaller degree of fluorescence quenching of pepsin compared to PFOA. Moreover, PFOA showed blue shift of the maximum emission wavelength, suggesting that PFOA could interact with pepsin, and that the fluorophore of protein was placed in a more hydrophobic environment after the addition of PFOA. Addition of PFNA resulted in the occurrence of an isoactinic point at 368 nm, indicating the existence of bound and frees PFNA in equilibrium system (Moreno and González-Jiménez, 1999). Their respective ability to bind with pepsin was different because of their different molecular structure.

3.2. Mechanism of fluorescence quenching

Fluorescence quenching usually are classified as dynamic quenching and static quenching (Geddes, 2001). The former is resulting from collisional encounters between the fluorophore and the quencher, while the static one is resulting from the formation of a ground-state complex between the fluorophore and the quencher. Furthermore, quenching mechanism can be distinguished by their dependence on temperature or by measuring the fluorescence lifetime (Xie et al., 2010). Further, the fluorescence quenching mechanism was analyzed using the Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + K_q\tau_0[Q] = 1 + K_{sv}[Q] \quad (2)$$

where F_0 and F represent fluorescence intensities of the pepsin in the absence and presence of the quencher, respectively. [Q] is the molar concentration of quencher; K_{SV} is the Stern-Volmer quenching constant; K_q is the bimolecular quenching constant, and τ_0 is average the lifetime of the fluorophore without quencher and is usually around 10^{-8} s (Lakowicz, 2006).

PFOA/PFNA showed linear Stern–Volmer plots at different temperatures (Fig. S1). Thus, K_{SV} of pepsin with PFOA/PFNA could be calculated, the corresponding K_{SV} at different temperatures were shown in Table 1. The K_q values of PFOA/PFNA were about the order of 10^{11} M^{-1} s^{-1}, if K_q is greater than 10^{10} M^{-1} s^{-1}, usually static quenching is occurring. Accurate result could be obtained through the data from time-resolved spectroscopy and will be explained inmore detail later in this article. Moreover, K_{SV} decreased with increasing temperature; hence, the quenching of pepsin fluorescence by PFOA/PFNA was the static one (Dong et al., 2014).

The binding constant and the number of binding sites for the complex formed between pepsin and PFOA/PFNA were estimated using the Scatchard equation:

$$r/D_f = nK - rK \quad (3)$$

where r is the number of moles of small molecule bound per mole of protein, D_f is the concentration of free small molecule, K is the binding constant, and n is the number of binding sites. As shown in

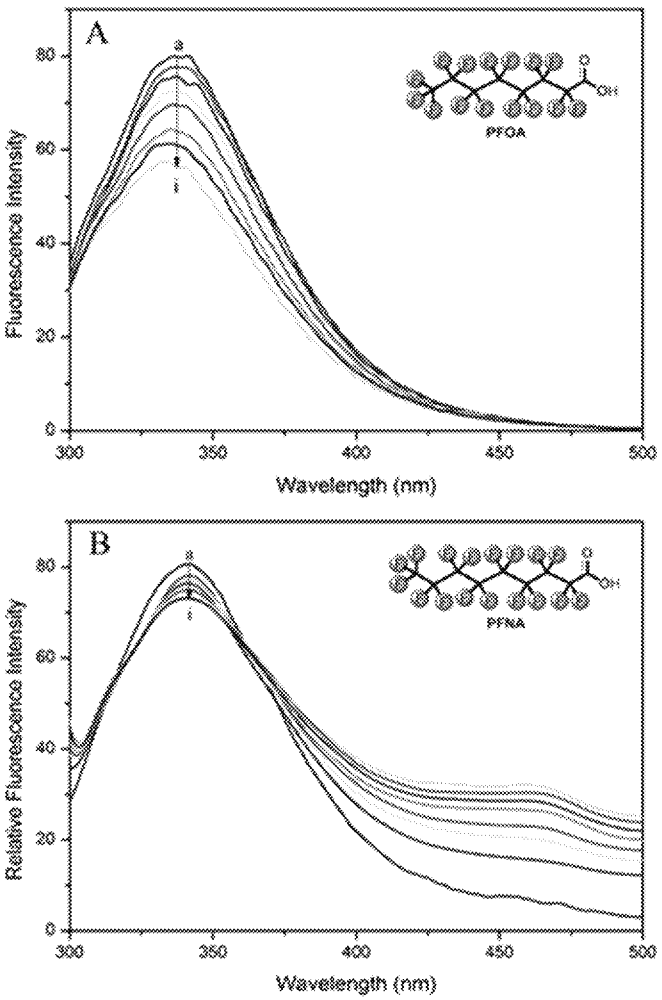

Fig. 1. Fluorescence titration of pepsin with increasing concentration of PFOA (A) or PFNA (B). [pepsin] = 3.0×10^{-6} M; [PFOA] = (a–i) (0–38.46) × 10^{-6} M; [PFNA] = (a–i) (0–38.46) × 10^{-6} M.

Fig. S1, Scatchard plots were plotted based on Eq. (3). The binding constant for PFOA was found to be 0.717×10^4 at 298 K. As a comparison, when a Scatchard plot analysis was performed for the binding between the PFNA and pepsin, the value of K obtained was lower (0.548×10^4). PFOA bound more strongly than PFNA indicating the higher affinity for complexation of PFOA over PFNA with pepsin. The value of K is of the order of 10^4 L mol^{-1}, indicating that a weak interaction exists between PFOA/PFNA and pepsin compared to known micromolecule, e.g. procyanidin B3-pepsin (0.961×10^4 M^{-1}) and baicalein (5.81×10^4 M^{-1}) (Li and Geng, 2016; Zeng et al., 2015). A linear Scatchard plots is generally indicative of one binding site in pepsin for PFOA/PFNA.

Time-resolved spectroscopy has proven to be an essential tool to gain information on the structure and dynamics of biomolecules on the nanosecond time scale. The application of the fluorescence lifetimes can be an efficient way to describe the quenching processes of simple model systems. Static quenching is a process of format static ground-state complexes, and not decrease the decay time of the uncomplexed fluorophores. The dynamic quenching is acting on the entire excited-state population, and thus decreases the mean decay time of the entire excited-state population. The decay profiles were presented in Fig. 2 and the fluorescence decay fitting parameters for the interaction of PFNA/PFOA and pepsin were collected in Table S1. We used biexponential approximation to

evaluate adequately the experimental data. The average decay time <τ> was calculated using the following equation:

$$(\tau) = \frac{\sum \alpha_i \tau^2}{\sum \alpha_i \tau} \quad (4)$$

where τ_i are the lifetimes components of the approximated decay curves; α_i are the pre-exponential factors. The average lifetime of pepsin in the absence and presence of 40 μM PFNA/PFOA changing from 6.11 ns to 6.08/6.10 ns? Based on the result, K_q at different temperatures were shown in Table 1. The K_q values of tested PFOA/PFNA were in the range of 2.86×10^{11}–3.86×10^{11} M^{-1} s^{-1}. Data in Table S1 indicated that the average lifetime did not experience significant variations with the presence of PFNA/PFOA, confirming a static quenching mechanism. This result coincided well with the fluorescence quenching data at different temperatures.

3.3. Evaluation of the thermodynamic parameters

The PFOA/PFNA consist of -C=O and -F groups, which can bind with the amino acid residues of pepsin by different acting forces. Such as the hydrogen or the hydrophobic interactions between the -C=O of PFOA/PFNA and pepsin interactions may be involved in the binding interactions of PFOA/PFNA with pepsin. Therefore, we have calculated using Van't Hoff equation which is a common method for the determination of the thermodynamic parameters. The thermodynamic parameters of binding reactions, enthalpy change (ΔH°) and entropy change (ΔS°), are the main evidence for confirming binding modes as well as the mechanism of binding (Ross and Subramanian, 1981). Details of thermodynamic parameter have been listed in Table 1. ΔG° is negative for both PFCs, which means that the binding progress of PFCs with pepsin were spontaneous. For the binding of the PFOA/PFNA, the positive values of ΔS° were the main contribution of the source of ΔG°, indicating that the PFOA/PFNA interacted with pepsin via hydrophobic interaction (Huang et al., 2010). The PFOA/PFNA bound through H-bonding interaction, as the sign of ΔH° is negative. The larger positive values of ΔS° in the case of PFNA compared to PFOA indicated that the PFNA binding interaction was hydrophobic by entropy driven. For the PFOA molecule binding occurred through hydrophobic interaction.

3.4. Effect of PFOA/PFNA on pepsin conformation

UV–vis absorption is a powerful technique to study the conformational analysis of small molecule-protein interactions. It is well known that pepsin has many aromatic amino acids, it gives an absorption peak at about 278 nm coming from π–π* electronic transition of aromatic rings (Tao et al., 1995). The UV–visible absorption spectra of pepsin showed a weak absorption peak at 276 nm in Fig. S2. The presence of PFOA or PFNA resulted in the absorption peak of pepsin at 276 nm decreases. The decrease in intensity of absorbance may be ascribed to the penetration of PFOA or PFNA into pepsin which restricts the mobility of PFOA or PFNA, resulting in the complex formation.

To elucidate the mode of interaction between PFNA/PFOA and pepsin, we conducted FT-IR studies. The FT-IR spectra of pepsin, [pepsin+PFOA]-PFOA and [pepsin+PFNA]-PFNA, were shown in Fig. 3. The frequency at 1650 cm^{-1}, which is amide I band, corresponds to $\upsilon_{C=O}$ vibration. The frequency at 1546 cm^{-1}, which is amide II band, corresponds to $\upsilon_{C=N}$ vibration. As Fig. 3 shows, the observed decrease in intensity and shift of the amide I and amide II band indicated that the reducing of α-helical structure in pepsin occurred in the presence of PFOA/PFNA. These findings validate a great deal of previous research on conformity.

Three-dimensional fluorescence spectroscopy is a rapid and

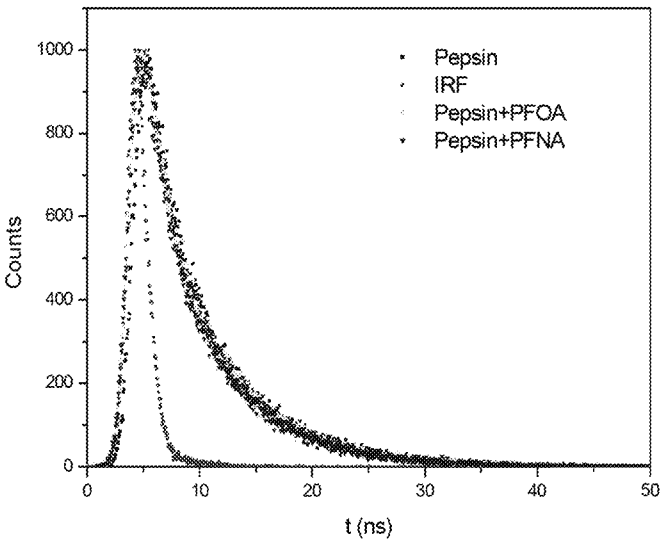

Fig. 2. The time resolved fluorescence decay profiles of pepsin in absence and presence of PFOA/PFNA at λ_{ex} = 280 nm [pepsin] = 3.0×10^{-5} M; [PFOA] = 6.0×10^{-5} M; [PFNA] = 6.0×10^{-5} M.

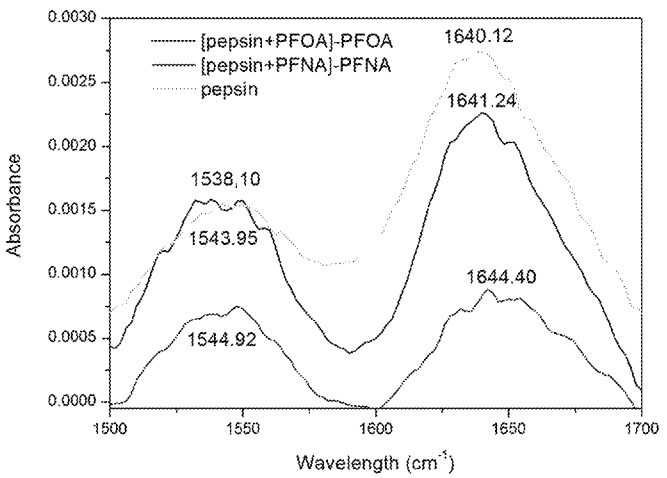

Fig. 3. FT-IR spectra of free pepsin (3.0×10^{-5} M), [PFOA] = 6.0×10^{-5} M, [PFNA] = 6.0×10^{-5} M.

Table 1
Values of binding parameters and thermodynamic parameters for binding of PFOA or PFNA with pepsin at different temperature.

System	T (K)	K_{sv} (× 10^5 L mol^{-1})	K_q (× 10^{13} L mol^{-1} s^{-1})	R^a	K/(L mol^{-1})	ΔG^0/(kJmol^{-1})	ΔS^0/(Jmol^{-1} K^{-1})	ΔH^0/(kJmol^{-1})	n
PFOA-pepsin	298	0.0236	0.0386	0.9993	0.0717×10^5	−21.96	21.66	−1.55	1.003
	310	0.0187	0.0306	0.9997	0.0563×10^5	−22.26			1.009
PFNA-pepsin	298	0.0180	0.0294	0.9992	0.0548×10^5	−21.33	66.23	−1.59	1.002
	310	0.0175	0.0286	0.9996	0.0534×10^5	−22.12			0.999

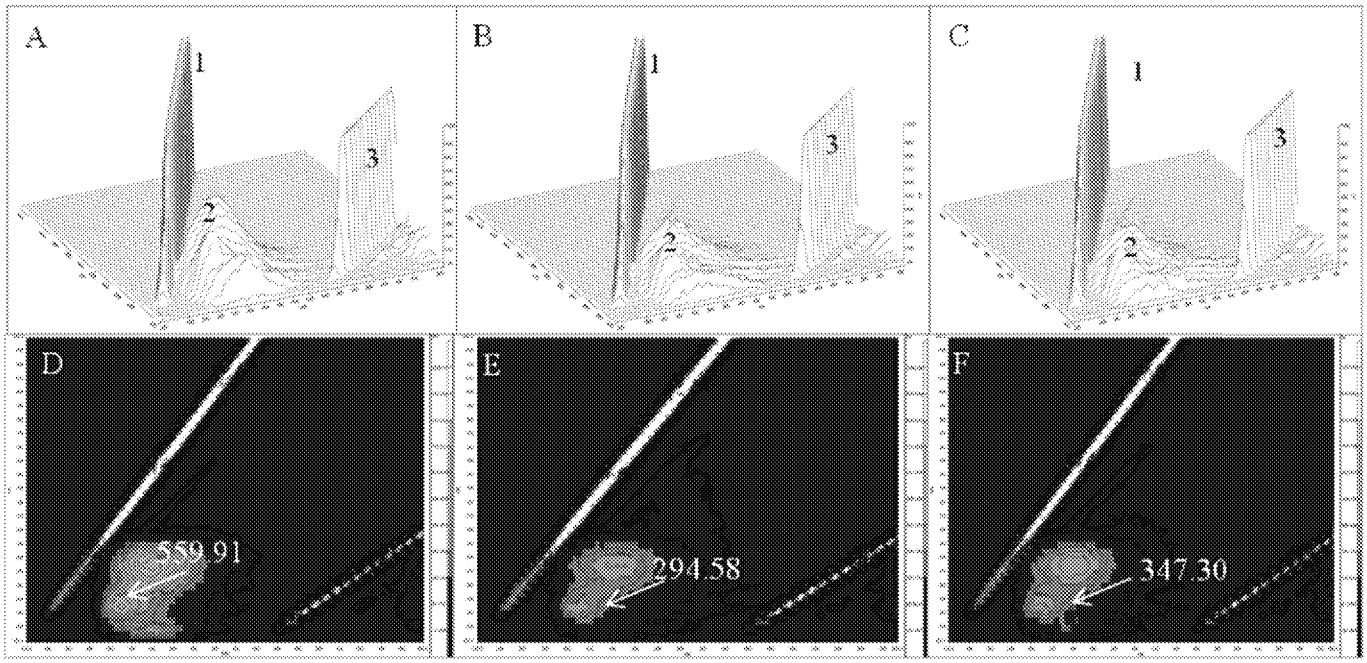

Fig. 4. The three-dimensional fluorescence spectra of pepsin (A), PFOA-pepsin systems (B) and PFNA-pepsin systems (C); the contour spectra of pepsin (D), PFOA-pepsin systems (E) and PFNA-pepsin systems (F); [pepsin] = 3.0×10^{-6} M, [PFOA/PFNA] = 6.0×10^{-6} M.

sensitive technique to provide information about the conformational and micro-environmental changes of protein (Ghosh et al., 2015; Wang et al., 2015). The representative three-dimensional fluorescence spectra (contours) belonging to pepsin, pepsin-PFOA, and pepsin-PFNA are shown in Fig. 4. There are three fluorescence peaks for pepsin, pepsin-PFNA, and pepsin-PFOA, peak 1 is the Rayleigh scattering peak ($\lambda_{em} = \lambda_{ex}$); peak 2 located at $\lambda_{em}/\lambda_{ex} = 280/340$ nm mainly reveals the spectral feature of Tyr and Trp residues; peak 3 is the second-ordered scattering peak ($\lambda_{em} = 2\lambda_{ex}$) (Zaroog and Tayyab, 2012). It is also observed from Fig. 4 that after the addition of PFOA/PFNA, the fluorescence intensity of both peak 2 peaks decreased. The relative fluorescence intensity of pepsin was changed from 559.91 to 294.58 for PFOA; from 559.91 to 347.30 for PFNA. These results demonstrated the interactions between PFOA/PFNA and pepsin induced conformation changed in pepsin (Wang et al., 2013).

The change in the secondary structure of pepsin was analyzed by CD spectra. The CD spectra of pepsin and pepsin-PFOA/PFNA mixture were shown in Fig. 5. The CD spectrum of pepsin had a single minimum at about 200 nm and no positive band, which indicated that pepsin featured a rich β-sheet conformation structure. After PFOA/PFNA added, the shape of spectra did not change significantly, but the intensity of the negative peak at 210 nm decreased. These results indicated that the PFOA/PFNA induced the disruption of the secondary structure. The β-sheet content of pepsin decreased on interaction with PFOA to a greater extent than PFNA and the possible reasons may be that the different numbers of -F groups in PFCs. PFOA and PFNA have different hydrophobic properties because their difference in molecular structure, and that resulted in the different ability of inducing secondary structure of pepsin. CD spectrum was further analyzed for the contents of different secondary structures using the package CDNN. The data in Table S2 suggested that the percentage of β-sheet in pepsin was higher (51.6%) compared to pepsin interact with PFOA (45.8%) or PFNA (48.9%). From Table S2 it is clear that reduction of α-helical structure is more in the case of PFOA compared to that for PFNA.

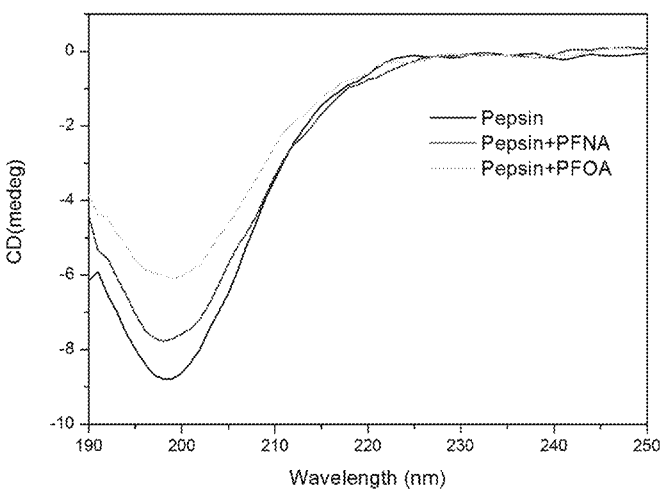

Fig. 5. Effect of PFOA/PFNA on intrinsic CD spectrum of pepsin, [pepsin] = 3.0×10^{-5} M, [PFOA/PFNA] = 6.0×10^{-5} M.

The damage to the structure of pepsin was attributed due to PFOA/PFNA's influence. The decrease of the α-helical and β-sheet content and concomitant increase of the β-turn and random coil structure indicated unfolding of native pepsin structure. The data also proposed that the PFOA/PFNA induced pepsin to undergo conformation changes at secondary structure levels, and this was mainly resulted from hydrophobic interactions (Zhu et al., 2012; Guercia et al., 2016).

3.5. Molecular docking

The molecular docking study was employed to identify the probable binding location of PFOA/PFNA within pepsin. The optimum pose with the lowest interaction energy between PFOA/PFNA and pepsin was shown in Fig. 6 (A and B), and the 2D ligand

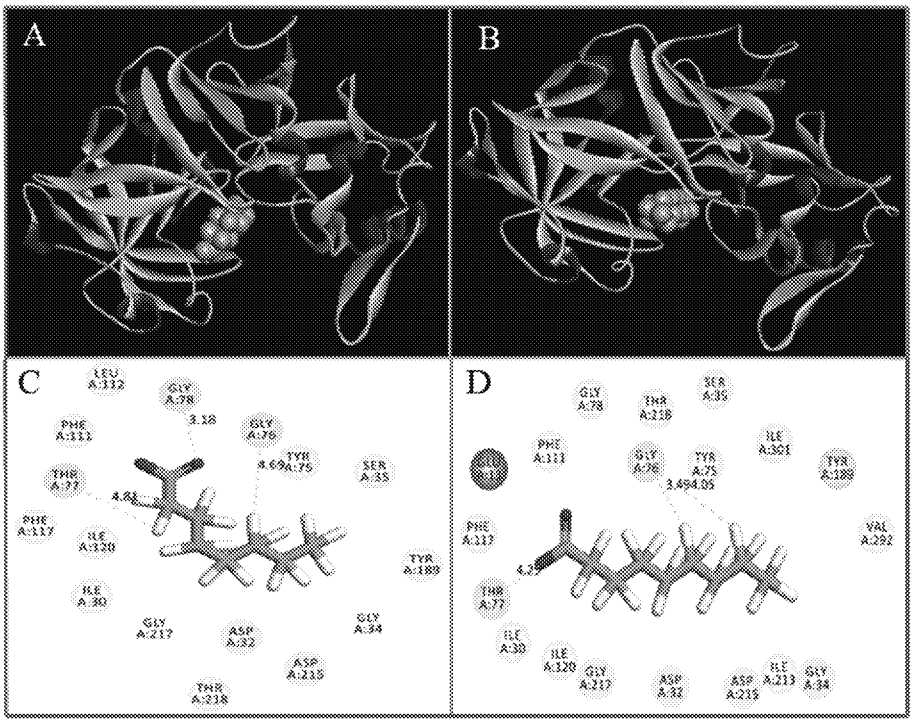

Fig. 6. Stereoview of the docked conformations of PFOA/PFNA-pepsin systems: PFOA-pepsin (A) and PFNA-pepsin (B). The pepsin was represented as a cartoon. The ligand structure was represented by a yellow one. Two-dimensional representation of PFOA (C) and PFNA (D) with interacting residues of pepsin. Polar and nonpolar residues were represented in the red and green circles, respectively. Green dashed line indicates hydrogen bond interactions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

interaction diagram was shown in Fig. 6 (C and D). Results showed the number of amino acid residues taking part in the binding interactions of PFOA with pepsin was 16; however, that of PFNA with pepsin was 19. Docking studies revealed that there were hydrogen bonds between carbonyl groups of PFOA and Gly78 of pepsin; -F group with Gly76 and Thr77, with a distance in the range of 3.18–4.71 Å. For PFNA, -O- group with Thr77; -F group with Gly76 and Tyr75, with a distance in the range of 3.49–4.25 Å. In addition, the hydrophobic interactions between the PFNA/PFOA and hydrophobic amino acid residues were also involved in these binding, such as Leu112, Phe111, Phe117, Gly76, Gly78, Gly217, Ile 30, Ile 120 and Ile 301. Corresponding binding free energy (ΔG^0) was a calculated as −19.21 kcaL/mol for PFOA, −18.67 kcaL/mol for PFNA. Its possible causes were the difference between crystal structure of pepsin and in solution. Finally, these results could clarify the existence of hydrophobic interaction between PFOA/PFNA and pepsin binding sites.

4. Conclusion

Due to the growing use of PFCs in the current society, that human being can contact with PFCs easily. Thermodynamic parameters indicated the binding of the PFOA/PFNA with pepsin was favored by hydrophobic forces. PFOA had stronger binding ability to pepsin than PFNA depending on their molecular structures. The analysis of FT-IR, 3D fluorescence, UV-vis demonstrated that the interaction between PFOA/PFNA and pepsin had effect on the secondary structure of the protein. The quantitative analyses of CD spectra supported that view. Docking results indicated that PFOA and PFNA could bind to pepsin with a similar binding domain. Our results may help explain the mechanism of PFCs affecting the conformation of digestive proteases, efforts to ensure environmental safety.

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Appendix ASupplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.07.047.

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